Molecular Biology of the Cell
Vol. 6, 119–134, January 1995

Cell Cycle Regulation of a *Xenopus* Wee1-like Kinase

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Submitted October 21, 1994; Accepted December 5, 1994
Monitoring Editor: Tim Hunt

Using a polymerase chain reaction-based strategy, we have isolated a gene encoding a Wee1-like kinase from *Xenopus* eggs. The recombinant *Xenopus* Wee1 protein efficiently phosphorylates Cdc2 exclusively on Tyr-15 in a cyclin-dependent manner. The addition of exogenous Wee1 protein to *Xenopus* cell cycle extracts results in a dose-dependent delay of mitotic initiation that is accompanied by enhanced tyrosine phosphorylation of Cdc2. The activity of the Wee1 protein is highly regulated during the cell cycle: the interphase, underphosphorylated form of Wee1 (68 kDa) phosphorylates Cdc2 very efficiently, whereas the mitotic, hyperphosphorylated version (75 kDa) is weakly active as a Cdc2-specific tyrosine kinase. The down-modulation of Wee1 at mitosis is directly attributable to phosphorylation, since dephosphorylation with protein phosphatase 2A restores its kinase activity. During interphase, the activity of this Wee1 homolog does not vary in response to the presence of unreplicated DNA. The mitosis-specific phosphorylation of Wee1 is due to at least two distinct kinases: the Cdc2 protein and another activity (kinase X) that may correspond to an MPM-2 epitope kinase. These studies indicate that the down-regulation of Wee1-like kinase activity at mitosis is a multistep process that occurs after other biochemical reactions have signaled the successful completion of S phase.

INTRODUCTION

All eukaryotic cells must replicate their DNA during S phase and distribute the resulting identical copies of their genetic information to daughter cells at mitosis. In most cells, the processes of chromosomal replication and segregation must be both coordinated with overall cellular growth and integrated with regulatory pathways that ensure the integrity of the genome. Cell cycle events such as S phase and mitosis are triggered by a large family of cyclin-dependent kinases (Cdks) that consist of a cyclin regulatory subunit and a Cdk catalytic partner. In turn, the cyclin-dependent kinases are controlled by a variety of regulatory factors, many of which govern the state of Cdk phosphorylation. These Cdk regulators must directly or indirectly respond to factors that control various checkpoints such as those involved in the monitoring of cell size, genome integrity, and mitotic spindle assembly [for reviews, see Hartwell and Weinert (1989), Nurse (1990), Solomon (1993), and Dunphy (1994)].

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At the G_2/M transition, a Cdk-cyclin complex known as maturation promoting factor (MPF) induces mitosis by phosphorylating a variety of structural and regulatory proteins that control mitotic processes such as chromosome condensation, spindle assembly, and nuclear envelope breakdown. MPF consists of the Cdc2 protein kinase (the archetypal member of the Cdk family) and a number of B-type cyclins (Dunphy *et al.*, 1988; Draetta *et al.*, 1989; Gautier *et al.*, 1988; Murray and Kirschner, 1989). The activation of MPF is controlled both translationally by the accumulation of the cyclin protein and posttranslationally by three known phosphorylations of the Cdc2 subunit. An enzyme known as CAK (Cdk-activating kinase) must phosphorylate Cdc2 on Thr-161 for acquisition of MPF activity but concomitant, dominantly inhibitory phosphorylations on Tyr-15 and Thr-14 serve to suppress the kinase activity of MPF during interphase [reviewed in Draetta (1993), Pines (1993), Solomon (1993)]. The Wee1 protein kinase phosphorylates Cdc2 on Tyr-15 but apparently not Thr-14 (McGowan and Russell, 1993; Parker and Piwnica-Worms, 1992; Kornbluth *et al.*, 1994). At the onset of mitosis, a highly

regulated phosphatase called the Cdc25 protein activates MPF by dephosphorylating Tyr-15 and most probably Thr-14 (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991).

A variety of studies have suggested that both the Cdc25 and Wee1 proteins are highly regulated during the cell cycle. In the case of the Cdc25 protein, much is known about the molecular mechanisms that control its Cdc2-specific tyrosine phosphatase activity. Specifically, a stimulatory kinase activity phosphorylates the N-terminal regulatory domain of the Cdc25 protein at the G_2/M transition, thereby allowing the C-terminal catalytic domain to dephosphorylate Cdc2 (Kumagai and Dunphy, 1992; Izumi *et al.*, 1992; Clarke *et al.*, 1993; Hoffman *et al.*, 1993). Considerably less is known about Wee1 regulation. Indirect measurements have indicated that Wee1 activity is high during interphase and low during mitosis, and experiments with heterologous in vitro systems have provided evidence that the inactivation of Wee1 at mitosis is due to phosphorylation (Solomon *et al.*, 1990; Smythe and Newport, 1992; Devault *et al.*, 1992; Tang *et al.*, 1993). However, the extent to which the modulation of Wee1 activity contributes to the mitotic-decision mechanism remains unresolved. In addition, the relationship between Wee1 regulation and the various cell cycle checkpoints, such as the one that monitors unreplicated DNA, is an important issue. These problems can be addressed best by examining the regulation of Wee1 kinase activity throughout the cell cycle in a homologous system. The *Xenopus* system is well suited for this purpose in that extracts from *Xenopus* eggs perform the cell cycle in vitro [reviewed in Murray (1991)]. Moreover, it has been possible to prepare *Xenopus* egg extracts containing unreplicated DNA that remained arrested in interphase due to the action of one or more inhibitory, replication-checkpoint factors (Dasso and Newport, 1990; Kornbluth *et al.*, 1992; Kumagai and Dunphy, 1992).

In this report, we describe the cloning and functional characterization of a Wee1-like kinase present in *Xenopus* eggs. With peptide-specific antibodies, we have isolated *Xenopus* Wee1 from egg extracts and measured its kinase activity and phosphorylation state at various points in the cell cycle. The kinase activity of *Xenopus* Wee1 is highly regulated during the cell cycle by phosphorylation, but the presence of unreplicated DNA during interphase does not appear to affect its activity or phosphorylation state to any measurable extent. We present evidence that the Wee1 protein is phosphorylated by multiple kinases at the G_2/M transition when it undergoes a large reduction in its ability to phosphorylate Cdc2. Since one of these Wee1-specific kinases corresponds to Cdc2, it appears that the down-modulation of Wee1 at mitosis involves in part a self-perpetuating loop where some active Cdc2 helps to trigger the full activation of MPF. In addition, the other Wee1-specific kinase (kinase X) may play a crucial role in mitotic initiation.

MATERIALS AND METHODS

cDNA and Library Production

Total RNA was prepared from de-folliculated mature *Xenopus* oocytes by a variation of the method of Chomczynski and Sacchi (1986). After the initial extraction with acidic phenol, the sample was clarified by centrifugation (16,000 × *g* for 10 min). The soluble fraction was extracted two times with 49% phenol/49% chloroform/2% isoamyl alcohol and then further purified by centrifugation through a 5.7 M CsCl cushion (Chirgwin *et al.*, 1979) with subsequent extractions and precipitations as described by Mueller *et al.* (1988). Poly(A)$^+$ RNA was prepared as described (Sambrook *et al.*, 1989) except that sodium dodecyl sulfate (SDS) was omitted from the elution buffer.

cDNA was synthesized by a variation of the hemimethylated protection method described by Palazzolo *et al.* (1990). First strand synthesis was performed at 42°C with poly(A)$^+$ *Xenopus* oocyte RNA and Superscript reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) in the presence of RNasin (Promega, Madison, WI), 5-methyl dCTP (Novagen, methylation dNTP mix), and a *Xho*I/oligo dT primer (Stratagene, La Jolla, CA). Second strand synthesis was performed at 15°C by using *E. coli* DNA polymerase I (New England BioLabs, Beverly, MA), ribonuclease H (Promega), and *Escherichia coli* DNA ligase (Life Technologies, Inc.) in the presence of normal dNTPs (Pharmacia Biotech Inc., Piscataway, NJ). Doubled-stranded cDNA was blunt-ended with T4 DNA polymerase (New England BioLabs) at 15°C before *Apa*I linkers (New England BioLabs) were added by T4 DNA ligase (New England BioLabs). Finally, the cDNA was digested with *Apa*I and *Xho*I, leaving the 5′ and 3′ ends of each insert flanked by *Apa*I and *Xho*I sticky ends, respectively. Since neither restriction enzyme cleaves hemimethylated DNA efficiently, internal *Apa*I and *Xho*I sites of the cDNA remain undigested. The cDNA was then used either for degenerate polymerase chain reaction (PCR) (see below) or directionally ligated into the cloning vector pAX-SV40 (P. R. Mueller and W. G. Dunphy, unpublished results) that had been digested with *Apa*I and *Xho*I. The cDNA/vector ligation reaction was transformed into the methylcytosine tolerant DH10B strain of *E. coli* (ElectroMAX DH10B, Life Technologies, Inc.) by electroporation, and ultimately 4.1×10^6 transformants where recovered by carbenicillin selection on 15-cm LB plates. Approximately 6.3×10^4 colonies/plate where grown to an average colony size of 0.5 mm before they were aseptically pooled from all plates by scraping into growth media. The library-transformed bacteria were then frozen in aliquots of 15% glycerol and stored at −80°C.

Isolation of a cDNA Encoding a Xenopus Wee1-like Protein Kinase

An internal *Xenopus* Wee1 fragment was cloned by PCR amplification using degenerate primers specific to regions of high amino acid sequence similarity shared between the *Schizosaccharomyces pombe* Wee1, *S. pombe* Mik1, and human Wee1 proteins (see Figure 1B). The 5′ primer was CGCGGATCC(C/T)(T/A)IGT(I/C)CA(C/T)(A/C/T)TIGA(C/T)(I/C)T(I/C)AA(A/G)CC and the 3′ primer was TCCCCCGGGTGCCAI(T/G/C)II(T/A)(C/G)ICC(G/A)TT(I/C)(C/T)(G/T/C)(I/C)GG. The 5′ end of each primer contains nine extra nucleotides (underlined) that provide restriction sites for *Bam*HI or *Sma*I. PCR reactions (50 μl) contained 20 ng *Xenopus* oocyte cDNA as template, 150 pmol of each primer, dNTPs, and buffer conditions recommended by the manufacturer. The reactions were heated to 85°C, and 1.25 units *Taq* DNA polymerase (AmpliTaq, Perkin-Elmer, Norwalk, CT) were added. Next, the reactions were heated to 94°C for 2.5 min followed by 95°C for 0.5 min. The reactions were then cycled 35 times at: 94°C for 1 min, 52°C for 2 min, and 72°C for

2 min with 5 s added to the extension phase of each successive cycle. In addition, an extra 5 min were added to the last 72°C extension step. An aliquot (20 μl) of the reaction was run on a 2% low melting point agarose gel, and an ~320-bp DNA band was isolated and subcloned into the TA II cloning vector (Invitrogen, San Diego, CA). DNA sequencing of 15 clones revealed one class of insert that had strong sequence similarity to the *S. pombe* Wee1, *S. pombe* Mik1, and human Wee1 proteins. This fragment was radiolabeled and used to screen the *Xenopus* oocyte cDNA library by colony hybridization (Sambrook *et al.*, 1989; Ausubel *et al.*, 1994). A full-length clone (pXe-Wee1) was selected for further study and sequenced on both strands by primer walking using Sequenase (U.S. Biochemical, Cleveland, OH) with the dideoxy chain termination method.

Plasmid Construction for Xenopus Wee1 Protein Production

To synthesize full-length *Xenopus* Wee1 recombinant protein, the initiation codon was converted to an *Nde*I restriction site by PCR. The 5′ PCR primer, CGGCAtATGAGGACGGCCATGTCATG, is complementary to the beginning of the open reading frame of *Xenopus* Wee1 (see Figure 1A) except that a single thymine (t, small case) was added. This extra base adds an *Nde*I site (underlined) to the initiation site. The 3′ PCR primer, AGCTTGAGCTTCTTCCATGTGCTG, is complementary to the region of *Xenopus* Wee1 immediately downstream of an unique *Kpn*I site (nucleotide 253, Figure 1A). PCR was performed using 200 ng of pXe-Wee1 in a 50-μl reaction under the conditions recommended by the manufacturer in the presence of 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer). The reactions were heated to 94°C for 2.5 min followed by 95°C for 0.5 min and then cycled 30 times at: 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min. In addition, an extra 7 min were added to the last 72°C extension step. The PCR reaction was used directly for subcloning into TA II (Invitrogen) as recommended by the manufacturer. The sequence of the modified *Xenopus* Wee1 fragment in the plasmid was confirmed by sequencing. This was then digested with *Nde*I and *Kpn*I to generate a 262-bp fragment containing the modified 5′ portion of *Xenopus* Wee1 (Xe-Wee1), which together with the ~1750 bp *Kpn*I/*Xho*I 3′ portion of Xe-Wee1, was ligated into either pET3a (Studier *et al.* 1990), pET3a-HIS (A. Kumagai and W. G. Dunphy, unpublished results), or pVL1393N-HIS (Tang *et al.*, 1993). The resulting vectors (pET-XeWee1, pET-HIS-XeWee1, and pVL-HIS-XeWee1) were used for preparation of *Xenopus* Wee1 protein (untagged or histidine-tagged, as indicated) in reticulocyte lysates, bacteria, or Sf9 insect cells.

Antibody Production

Rabbits were immunized with either a peptide corresponding to the C-terminal end of *Xenopus* Wee1 (VGAKNTRSLSFTCGGY) conjugated to keyhole limpet hemocyanin or purified, full-length *Xenopus* Wee1 protein expressed in bacteria (see below). Anti-peptide antibodies were purified by affinity chromatography on Affi-Gel-10 columns (Bio-Rad, Richmond, CA) containing covalently bound peptides. Antibodies against *Xenopus* Cdc2 (Milarski *et al.*, 1991) and fission yeast Wee1 (Tang *et al.*, 1993) were prepared as described. Antibodies against human cyclin B1 (GNS-1) and phosphotyrosine (4G10) were obtained from Pharmingen, San Diego, CA, and Upstate Biotechnology, Lake Placid, NY, respectively. The MPM-2 antibodies were a generous gift of J. Kuang.

Production and Purification of Proteins from Baculovirus-Infected Insect Cells and Bacteria

Sf9 insect cell lysates containing histidine-tagged *Xenopus* Wee1, histidine-tagged human cyclin B1 (A. Kumagai and W. G. Dunphy, unpublished data), wild type *Xenopus* Cdc2 (Coleman *et al.*, 1993), mutant *Xenopus* Cdc2 (T161A, T14A, or Y15F) (A. Kumagai and W. G. Dunphy, unpublished data), and histidine-tagged fission yeast Wee1 (Tang *et al.*, 1993) were prepared by established procedures (Desai *et al.*, 1992). *Xenopus* Cdc2-containing lysates were aliquoted, frozen in liquid nitrogen, and stored at −80°C. Histidine-tagged proteins were immediately bound to nickel-iminidoacetic acid (IDA) Sepharose (Sigma Chemical, St. Louis, MO) and either frozen directly in liquid nitrogen for later use or purified as described (A. Kumagai and W. G. Dunphy, unpublished data) except that in the case of histidine-tagged Wee1 protein the elution buffer contained 5 mM 2-mercaptoethanol. Proteins which were added to *Xenopus* egg extracts were dialyzed against 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-NaOH, pH 7.4, 150 mM NaCl, and 0.1 mM dithiothreitol.

A plasmid encoding histidine-tagged full-length *Xenopus* Wee1 protein (pET-HIS-XeWee1) was transformed into BL21(DE3)pLysS. These bacteria were grown to mid-log phase, induced, harvested, and lysed essentially as described (Kumagai and Dunphy, 1991) except induction was at 37°C and sonication was performed in 20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 5 mM 2-mercaptoethanol, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′tetraacetic acid (EGTA), and 0.1% Nonidet P-40. The inclusion body fraction was dissolved in 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, and 6 M urea. Following clarification (20 min at 30,000 × *g*), the urea-solubilized protein was purified by nickel-IDA Sepharose chromatography as described above. A highly enriched 68-kDa band corresponding to the *Xenopus* Wee1 protein was purified to homogeneity by electroeluting in an Elutrap (Schleicher & Schuell, Keene, NH). The pure protein was used as a standard to estimate the concentration of endogenous Wee1 protein in *Xenopus* extracts and for antibody production.

Assay for Xenopus Wee1-mediated Tyrosine Phosphorylation of Cdc2

Purified complexes between histidine-tagged human cyclin B1 and either wild type or mutant *Xenopus* Cdc2 were prepared as described (A. Kumagai and W. G. Dunphy, unpublished data). In some cases, *Xenopus* Cdc2 was isolated from insect cell lysates on agarose beads containing the fission yeast Suc1 protein (p13) at a concentration of 5 mg/ml (Dunphy *et al.*, 1988). The *Xenopus* Cdc2 protein was eluted from the beads by treatment with 0.15 mg/ml *Xenopus* Suc1 protein (our unpublished data) dissolved in lysis buffer. This purified Cdc2 protein was added to the eluted, histidine-tagged human cyclin B1 to yield to the Cdc2/cyclin B substrate. Control experiments indicated that the *Xenopus* Suc1 protein did not affect the Wee1 kinase assay.

Two in vitro kinase assays were developed to measure the kinase activity of the *Xenopus* Wee1 protein. In the first, *Xenopus* Wee1 (recombinant or immunoisolated from *Xenopus* extracts) was combined with the purified Cdc2/cyclin B substrate (either wild-type Cdc2 or the Cdc2T161A mutant) and an ATP regenerating system essentially as described (Coleman *et al.*, 1993) except that $MnCl_2$ was omitted from the kinase buffer and the reaction was performed at 21°C. Following this incubation, samples were processed for immunoblotting. In the second assay, *Xenopus* Wee1 was combined with the purified kinase-negative substrate (Cdc2T161A/cyclin B) in kinase buffer in the presence of [^{32}P]ATP (125 μCi/ml). In some cases, a 10-fold excess of non-radioactive ATP was included. Following a 15–20-min incubation at 21°C, samples were processed for autoradiography.

Xenopus Egg Extracts

Xenopus cytostatic factor (CSF)-arrested egg extracts (arrested in M phase) were prepared from unactivated eggs as described by Murray (1991). To drive these extracts into interphase and observe their cell cycle progression, $CaCl_2$ (0.4 mM) and demembranated *Xenopus* sperm nuclei (200/μl) were added. In some cases, extracts were arrested in interphase with either cycloheximide (100 μg/ml) or aphidicolin (50

μg/ml in the presence of 1000–2000 sperm nuclei per μl of egg extract).

Cytosolic and membrane fractions from either CSF-arrested or interphase-arrested (40 min post-activation) *Xenopus* egg extracts were prepared by diluting the extracts with 2 volumes of EB buffer (80 mM sodium β-glycerol phosphate, pH 7.3, 15 mM $MgCl_2$, 20 mM EGTA) containing 3 μM okadaic acid and protease inhibitors (10 μg/ml each of pepstatin, chymostatin, and leupeptin), and centrifuged at 260,000 × *g* for 1 h at 4°C. Membrane fractions were resuspended in EB (volume equal to cytosol).

Preparation of Cdc2-depleted CSF Cytosol

Cytosolic fractions of CSF-arrested *Xenopus* extracts were incubated with 0.1 volume of either control-agarose beads or p13-agarose beads (5 mg/ml bound p13) for 30 min at 4°C. Beads were removed by centrifugation (10 s at 1400 × *g*). To ensure quantitative Cdc2 removal, extracts were reincubated with fresh p13-agarose beads for a total of four times.

Isolation of Endogenous Cdc2 and Wee1 from Xenopus Extracts on p13-Agarose

Aliquots of a calcium-activated egg extracts were taken at various intervals and frozen in liquid nitrogen. Subsequently, these aliquots were thawed, diluted with 2 volumes of EB buffer containing 25 mM NaF and 1 mM Na_3VO_4, clarified by centrifugation (5 min, 16,000 × *g*) and incubated under constant agitation with 0.25 volume of p13-agarose beads (5 mg/ml bound p13) for 30 min at 4°C. Following this incubation, the beads were processed as described in (Solomon *et al.*, 1990) except that dithiothreitol was omitted from the washes.

Immunoisolation of Endogenous Xenopus Wee1 Protein

Nonionic detergent (Nonidet P-40, 1% final concentration) was added to aliquots of either CSF-arrested, interphase or S phase egg extracts. Next, these extracts were incubated with affinity purified anti-*Xenopus* Wee1 peptide antibodies (44 μg/ml) for 1 h at 4°C. Subsequently, protein A-agarose beads (0.1 volume) were added. Following rotation at 4°C for 1 h, the beads were washed successively with EB buffer containing 25 mM NaF, 1 mM Na_3VO_4, 0.1% Nonidet P-40, and 1 μM okadaic acid (once); EB buffer containing 25 mM NaF, 1 mM Na_3VO_4, 0.1% Nonidet P-40 (twice); EB buffer containing 25 mM NaF and 1 mM Na_3VO_4 (three times); and kinase buffer (twice). All buffers contained protease inhibitors as indicated above and ovalbumin (1 mg/ml final concentration). Finally, the immunoisolated Wee1 was either used directly or frozen in liquid nitrogen and stored at −80°C.

In Vitro Dephosphorylation of Hyperphosphorylated Xenopus Wee1 Protein

Antibody beads containing hyperphosphorylated Wee1 protein from M-phase extracts (see above) were washed successively in EB buffer containing ovalbumin and protease inhibitors (once) and phosphatase buffer (50 mM Tris-HCl, pH 7.5, 67 μM $CaCl_2$, 1.34 mM $NiCl_2$, 1 mg/ml ovalbumin; twice). The washed beads were then agitated in the presence of 0.4 unit/ml human erythrocyte protein phosphatase 2A (Upstate Biotechnology) in phosphatase buffer for 10 min at 21°C. Following this incubation, the phosphatase was inactivated with okadaic acid (4 μM final concentration) and the beads were washed with kinase buffer containing 1 mg/ml ovalbumin (twice). The phosphatase-treated Wee1 was immediately assayed for kinase activity using the cyclin/Cdc2 substrate described above.

In Vitro Translation of Xenopus Wee1 Protein

The pET3a-XeWee1 vector was linearized with *Xho*I and capped messenger RNA (mRNA) was prepared using T7 RNA polymerase and the mCAP kit (Stratagene). Full-length *Xenopus* Wee1 protein was translated from this synthetic mRNA in a rabbit reticulocyte lysate (Promega) in the presence of Tran^{35}S-Label (ICN Pharmaceuticals, Covina, CA).

Miscellaneous Methods

Histone H1 kinase assays were performed as described in Dunphy and Newport (1989). Phosphoamino acid analysis was carried out according to standard procedures (Boyle *et al.*, 1991). Protein concentrations were determined with the Bio-Rad protein assay kit. Immunoblotting was performed as described previously (Coleman *et al.*, 1993) using ^{125}I-labeled protein A (ICN) or ^{125}I-labeled sheep anti-mouse antibodies (Amersham, Arlington Heights, IL) except that 3% bovine serum albumin and 10% heat-inactivated bovine serum were used for the blocking agents for the anti-*Xenopus* Wee1 peptide and MPM-2 antibodies, respectively. Quantification of both H1 and Wee1 kinase assays was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Fluorography was performed using Amplify (Amersham).

RESULTS

Isolation of a Xenopus Wee1-like Kinase

To clone potential *Xenopus* Wee1 homologs, we utilized two degenerate oligonucleotides to amplify segments of *Xenopus* oocyte cDNA in a PCR as described in MATERIALS AND METHODS. These primers were designed to anneal with conserved sequences in catalytic subdomains VI and X of the Wee1 family (e.g., fission yeast Wee1 and Mik1 as well as human Wee1). PCR amplification yielded a ~320-bp DNA fragment encoding a protein sequence with significant homology to the Wee1 class of protein kinases. This fragment was then used to probe a *Xenopus* oocyte library for the full-length cDNA. The full-length gene for the *Xenopus* Wee1-like kinase contains an open reading frame of 555 amino acids corresponding to a polypeptide with a predicted molecular mass of 62 kDa (Figure 1A; GenBank accession no. U13962). In comparison with previously described members of the Wee1 family, *Xenopus* Wee1 is most similar to the human Wee1-like kinase (Igarashi *et al.*, 1991). The *Xenopus* Wee1 protein is 71% identical in its catalytic domain (Figure 1B) and 52% identical (68% similar) over its full coding sequence to human Wee1 (N. Watanabe, M. Broome, and T. Hunter, unpublished observations). In addition, both the human and *Xenopus* homologs contain a 19 amino acid insertion between catalytic subdomains VI and VII, and have a less common ANE sequence (instead of APE) in subdomain VIII. The catalytic domain of *Xenopus* Wee1 is also significantly related to the *S. pombe* Wee1 (35% identity) (Russell and Nurse, 1987) and Mik1 proteins (32% identity) (Lundgren *et al.*, 1991) as well as the *Saccharomyces cerevisiae* Swe1 kinase (32% identity) (Booher *et al.*, 1993). Finally, all of these five members of the Wee1 family share five residues (see asterisks in

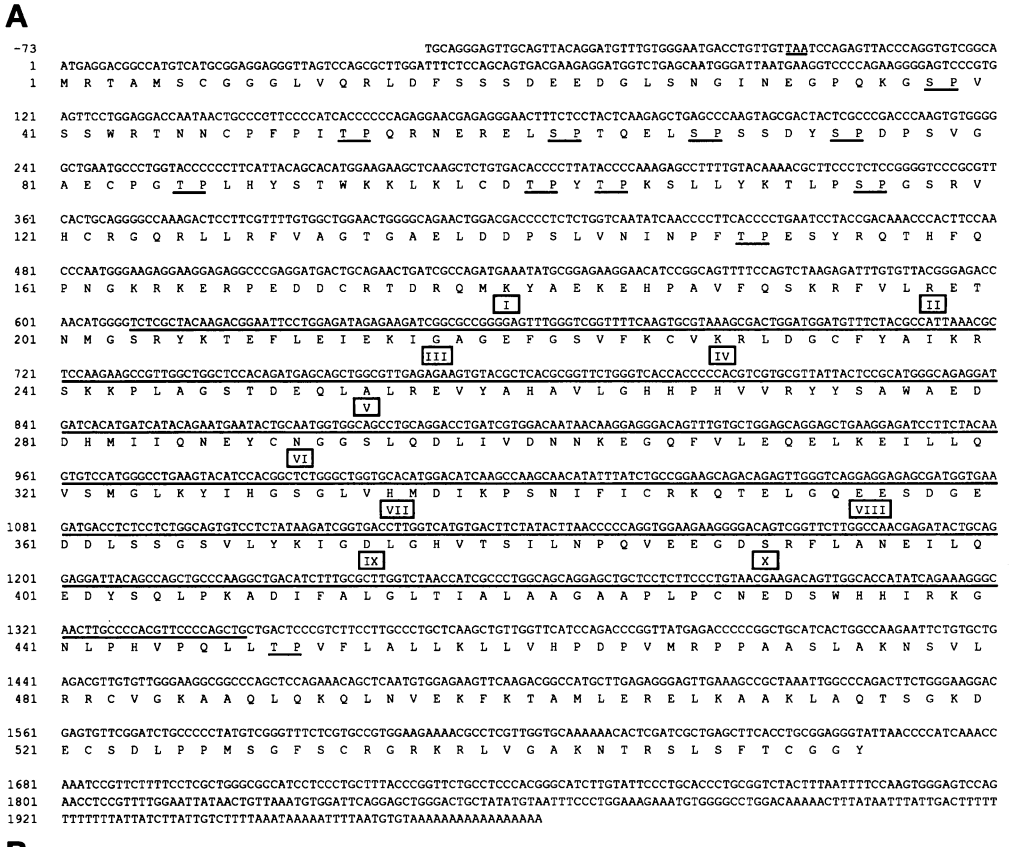

Figure 1. Sequence analysis of a *Xenopus* Wee1-like gene. (A) Nucleotide and predicted amino acid sequence of the *Xenopus* Wee1 gene. Nucleotide sequence of a ~2070-bp *Apa*I to *Xho*I fragment is shown. A predicted open reading frame of 555 amino acids is preceded by an in frame termination codon (TAA). The putative catalytic domain of *Xenopus* Wee1 is underlined. Roman numerals denote the catalytic subdomains found in eukaryotic protein kinases (Hanks, 1991). Ser/Thr-Pro motifs that are potential phosphorylation sites for a variety of mitotic kinases are underlined. (B) Alignment and comparison of the catalytic domains of various Wee1-like kinases: *Xenopus* Wee1 (Xe-Wee1, residues 210–448), Human Wee1 (Hu-Wee1, residues 187–432), *S. pombe* Wee1 (Sp-Wee1, residues 560–786), *S. pombe* Mik1 (Sp-Mik1, residues 283–510), and *S. cerevisiae* Wee1, SWE1 (Sc-Wee1, residues 438–673). Identical residues shared between two or more members of the Wee1 kinase family are boxed. Asterisks designate amino acids that are conserved between all known members of the Wee1 kinase family, but not in other eukaryotic protein kinases. Arrows indicate sequences that were used to design degenerate PCR primers. Sequence alignment was performed by the SEQVAX PILEUP program based on the method of Feng and Doolittle (1987).

Figure 1B) that are indicative of the Wee1 family, since these residues are not commonly found in other kinases (Hanks, 1991).

Xenopus Wee1 Phosphorylates Cdc2 on Tyrosine 15 in a Cyclin-dependent Manner

To establish the biochemical properties of *Xenopus* Wee1, we expressed a histidine-tagged version of the protein in baculovirus-infected insect cells (Figure 2A). To assess whether the baculovirus-expressed Wee1 protein was catalytically active, we first asked whether it was capable of autophosphorylation. Upon incubation with [^{32}P]ATP, the purified *Xenopus* Wee1 protein became strongly radiolabeled (Figure 2B). Phosphoamino acid analysis of the labeled Wee1 protein revealed the presence of mainly phosphotyrosine with a lesser amount of phosphoserine (Figure 2C).

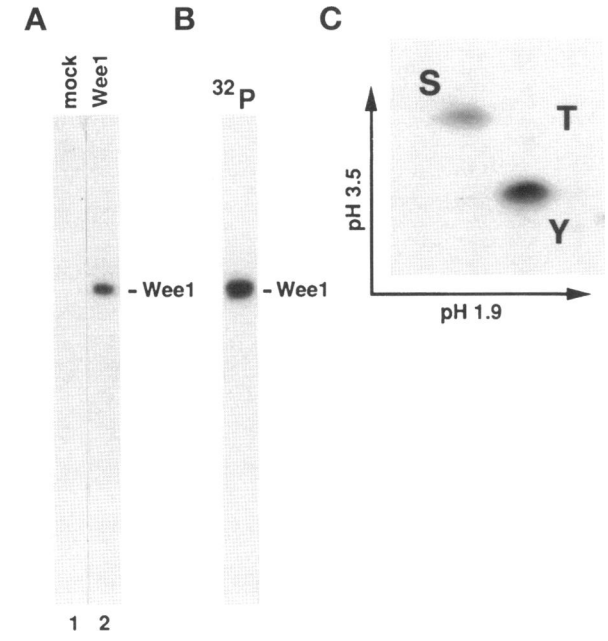

Figure 2. Characterization of baculovirus-expressed *Xenopus* Wee1 protein kinase. (A) Lysates from either uninfected Sf9 insect cells (mock, lane 1) or cells infected with a *Xenopus* Wee1-encoding baculovirus (Weel, lane 2) were immunoblotted with anti-*Xenopus* Wee1 peptide antibodies. (B) Purified recombinant *Xenopus* Wee1 was incubated with [^{32}P]ATP in vitro and processed for autoradiography. (C) Phosphoamino acid analysis of ^{32}P-labeled *Xenopus* Wee1 protein from (B) shows that it is predominantly phosphorylated on tyrosine (85%), with some labeling on serine (14%). S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

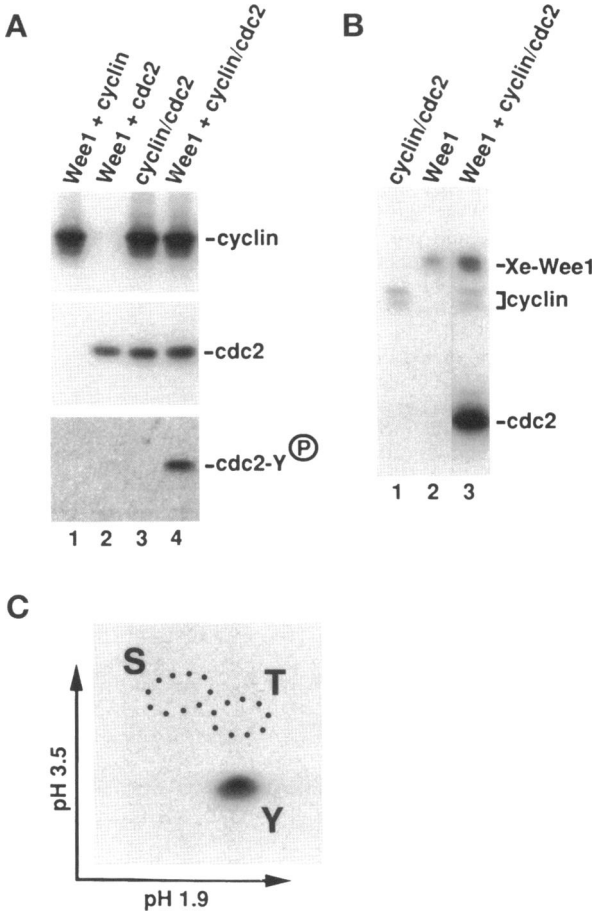

Figure 3. *Xenopus* Wee1 phosphorylates Cdc2 exclusively on tyrosine in a cyclin-dependent manner. (A) Purified recombinant proteins: *Xenopus* Wee1 (Wee1, lanes 1, 2, and 4), human cyclin B1 (cyclin, lanes 1, 3, and 4), and *Xenopus* wild-type Cdc2 (Cdc2, lanes 2, 3, and 4) were incubated in the presence of an ATP-regenerating system (see MATERIALS AND METHODS) and processed for immunoblotting using either anti-cyclin (top panel), anti-Cdc2 (middle panel), or anti-phosphotyrosine (bottom panel) antibodies. (B) Purified recombinant proteins: a complex of human cyclin B1 and *Xenopus* T161A kinase-negative Cdc2 (cyclin/Cdc2; lanes 1 and 3) and *Xenopus* Wee1 (Wee1; lanes 2 and 3) were incubated in the presence of [^{32}P]ATP. Following a 20 min incubation at 21°C, samples were electrophoresed and processed for autoradiography. (C) Phosphoamino acid analysis of Cdc2 from (B) shows that *Xenopus* Wee1 phosphorylates Cdc2 exclusively on tyrosine. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

To ask whether the recombinant Wee1 would phosphorylate its presumed physiological target, we utilized Cdc2 and cyclin B proteins that had been expressed in baculovirus-infected insect cells (A. Kumagai and W. G. Dunphy, submitted). Routinely, the Cdc2 and cyclin proteins were produced separately in insect cells. Next, the cyclin protein was purified on nickel-agarose by virtue of a six-histidine tag that had been engineered into its coding sequence. The cyclin-coated agarose beads were then added to Cdc2-containing insect cell lysates, and the resulting Cdc2-cyclin complex was isolated as described (A. Kumagai and W. G. Dunphy, unpublished data) (see MATERIALS AND METHODS). In some cases, the cyclin and Cdc2 proteins were purified separately before addition to the kinase assay (see MATERIALS AND METHODS). As shown in Figure 3A (lane 4), the incubation of the Cdc2/cyclin B complex with recombinant *Xenopus* Wee1 protein results in efficient tyrosine phosphorylation of the Cdc2 subunit. In this experiment, the tyrosine phosphorylation of Cdc2 was monitored by immunoblotting with anti-phosphotyrosine antibodies. As expected, no tyrosine phosphorylation of Cdc2 was detected if either the Cdc2 or Wee1 protein was omitted from the incubation (Figure 3A, lanes 1 and 3). Significantly, there was also no Wee1-catalyzed phosphorylation of Cdc2 if purified Cdc2 alone instead of the Cdc2/cyclin B complex, was employed as the substrate (Figure 3A, lane 2). This observation demonstrates that the recognition of Cdc2 by *Xenopus* Wee1 in vitro is cyclin-dependent, which is consistent with earlier experiments indicating that Cdc2 does not become tyrosine phosphorylated in cycloheximide-treated *Xenopus* egg extracts that lack cyclin B (Solomon *et al.*, 1990; Kumagai and Dunphy,

1991; Parker *et al.*, 1991). Interestingly, the kinase activity of *Xenopus* Wee1 is highly thermolabile: the protein loses essentially all of its activity upon a 10-min incubation at 36°C (our unpublished observation). This thermal instability may reflect the fact that *Xenopus* is a cold-blooded organism.

To assess whether *Xenopus* Wee1 would phosphorylate Cdc2 solely on tyrosine, we treated the Cdc2/cyclin complex with Wee1 protein in the presence of [^{32}P]ATP, and then subjected the resulting labeled Cdc2 protein to phosphoamino acid analysis (Figure 3, B and C). A technical consideration is that we used a kinase-inactive version of Cdc2 (the T161A mutant that cannot be phosphorylated at a site required for catalytic activity) to reduce the potential background incorporation of serine and threonine that might result from the presence of highly active Cdc2 protein kinase. Control experiments in which tyrosine phosphorylation of Cdc2 was measured with anti-phosphotyrosine antibodies indicated that *Xenopus* Wee1 phosphorylated wild-type and T161A mutant Cdc2 equally well (see Figure 4). Quantitation of the data from the phosphoamino acid analysis (Figure 3C) indicated that 99% of the radioactive phosphate was present in phosphotyrosine. Thus, *Xenopus* Wee1, like the human Wee1 homolog, phosphorylates Cdc2 only on tyrosine and apparently not on threonine residues such as Thr-14 (Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993).

To establish whether the tyrosine phosphorylation of Cdc2 occurred only on the physiologically relevant site (Tyr-15), we repeated the Wee1 assays using a variety of Cdc2 mutants as the kinase substrates (Figure 4). Specifically, we utilized the Cdc2 Y15F mutant that cannot be phosphorylated on Tyr-15 as well as the T161A and T14A mutant forms of Cdc2. As shown in Figure 4A, in experiments where both the T161A mutant and wild-type versions of Cdc2 were efficiently phosphorylated on tyrosine by Wee1 (Figure 4A, lanes 1–4 and 5–8), there was no detectable tyrosine phosphorylation of the Y15F mutant (Figure 4A, lanes 9–12). In similar experiments, the T14A mutant also was phosphorylated efficiently by Wee1, as might be expected (not shown). Taken together, these results indicate that *Xenopus* Wee1 phosphorylates Cdc2 only on tyrosine 15, and that recognition of Cdc2 by Wee1 is not appreciably dependent upon prior phosphorylation of either the threonine 161 or threonine 14 residues. Importantly, the tyrosine phosphorylation of wild-type Cdc2 also resulted in a substantial reduction of its activity as a histone H1 kinase (Figure 4B, middle columns). As expected, the Y15F mutant that had been treated with Wee1 showed no diminution in H1 kinase activity (Figure 4B, right columns), while the Wee1-treated T161A mutant was inactive as an H1 kinase whether or not it had been phosphorylated by Wee1 (Figure 4B, left columns).

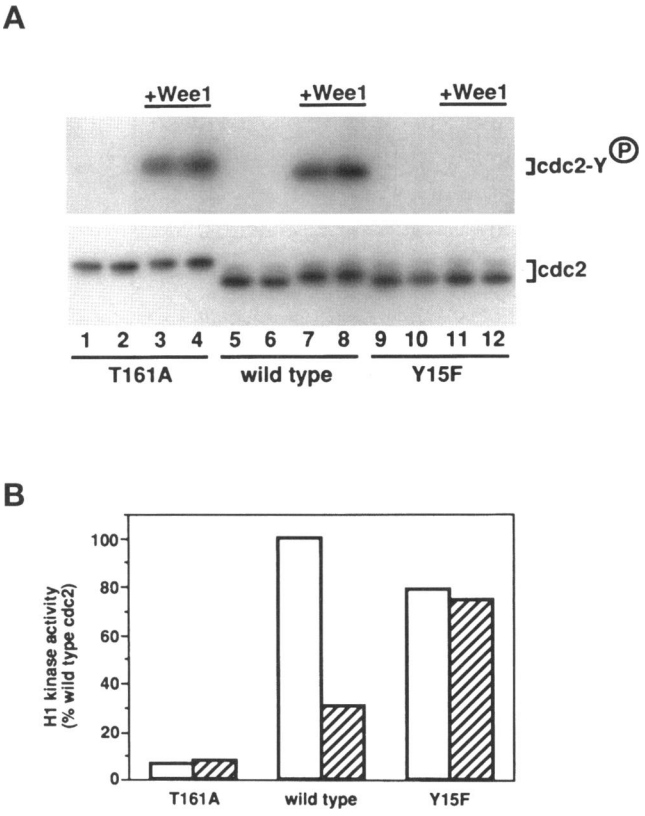

Figure 4. *Xenopus* Wee1 phosphorylates Cdc2 specifically on tyrosine 15 resulting in a decrease in the H1 kinase activity of the modified Cdc2/cyclin complex. (A) Purified recombinant human cyclin B1 bound to various forms of *Xenopus* Cdc2 (T161A, threonine 161 mutated to alanine, lanes 1–4; wild type, lanes 5–8; Y15F, tyrosine 15 mutated to phenylalanine, lanes 9–12) was used in in vitro kinase assays (see MATERIALS AND METHODS) that contained either control buffer (lanes 1, 2, 5, 6, 9, and 10) or purified *Xenopus* Wee1 (+Wee1, lanes 3, 4, 7, 8, 11, and 12). The samples were processed for immunoblotting with either anti-phosphotyrosine (top panel) or anti-Cdc2 (bottom panel) antibodies. (B) Cdc2-associated H1 kinase activity of the samples shown in part (A) in the presence (stripped bars) or absence (clear bars) of *Xenopus* Wee1. As expected, *Xenopus* Wee1-modified wild type Cdc2 loses much of its kinase activity (~30% of control-treated). In contrast, the H1 kinase activity of both the T161A form (which is inactive as a kinase) and the Y15F form (which lacks the only residue *Xenopus* Wee1 phosphorylates) were unchanged by incubation with *Xenopus* Wee1 (see text).

Exogenously Added Xenopus Wee1-like Kinase Causes a Mitotic Delay in Egg Extracts

In fission yeast, the length of the G_2 phase is directly correlated with the intracellular concentration of the Wee1 protein such that overexpression of Wee1 leads to a mitotic delay and inactivation of Wee1 leads to a shortening of the G_2 phase (Nurse, 1975; Russell and Nurse, 1987). To ask whether the concentration of the *Xenopus* Wee1 protein could influence the length of the cell cycle, we added varying amounts of exogenous Wee1 protein to cell cycle extracts from *Xenopus* eggs (Figure 5, A and B). We observed that the recom-

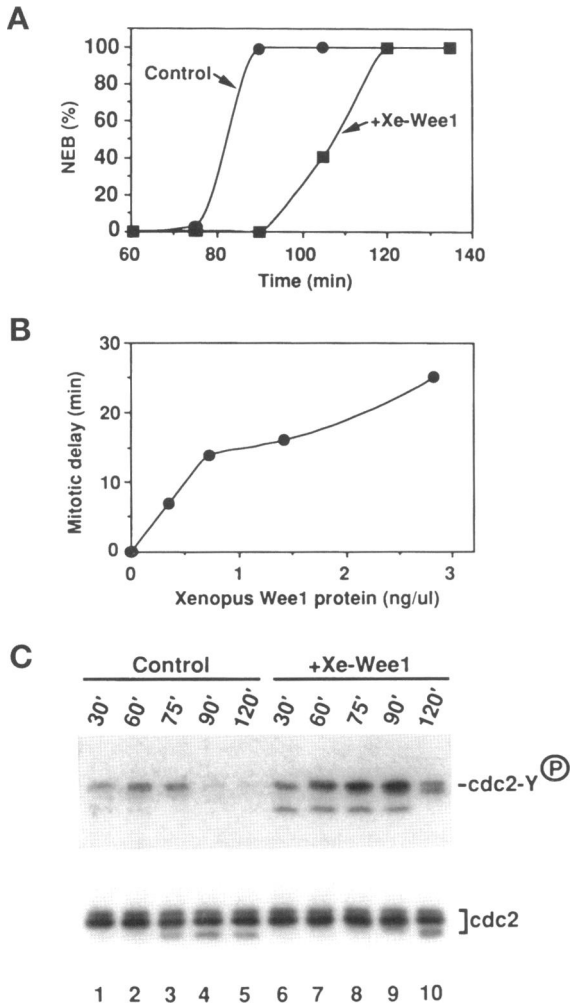

Figure 5. *Xenopus* Wee1 causes a dose-dependent mitotic delay in frog egg extracts and a concomitant increase in the phosphotyrosine content of Cdc2. (A) CSF-arrested *Xenopus* egg extracts were activated with $CaCl_2$ and incubated at 21°C. At t = 20 min, 0.2 volume of either control buffer (circles) or purified *Xenopus* Wee1 (squares) was added. At the indicated times, aliquots were removed and assessed for nuclear envelope breakdown (NEB) by visualizing with phase contrast microscopy and Hoechst staining. (B) Plot of mitotic delay (time difference of half-maximal NEB) between the control or Wee1-treated extracts vs. the added concentration of exogenous Wee1 protein. (C) Aliquots were taken from the incubation shown in part A at the indicated times (30–120 min). Endogenous Cdc2 was isolated with p13-agarose beads and subjected to immunoblotting with either anti-phosphotyrosine (top panel) or anti-Cdc2 (bottom panel) antibodies. In the control samples (lanes 1–5), Cdc2 undergoes tyrosine dephosphorylation between 75 and 90 min, the time at which the extract entered mitosis (part A). In contrast, the Wee1-treated extract (lanes 6–10) contains tyrosine phosphorylated Cdc2 for a prolonged period (>120 min), consistent with a delay in mitotic entry (part A).

binant *Xenopus* Wee1 protein elicited a dose-dependent delay of the entry into mitosis (Figure 5B). At the highest concentration of added Wee1 protein (3 ng/μl), there was a ~30 min delay in the time required for the extracts to enter mitosis, as judged by visual monitoring of nuclear disassembly. In these experiments, it was important to know the concentration of the endogenous Wee1 protein in the egg extracts. Using anti-*Xenopus* Wee1 antibodies and known amounts of bacterially expressed *Xenopus* Wee1 protein (see MATERIALS AND METHODS), we estimated that the endogenous level of the Wee1 protein is ~1 ng/μl. Thus, the addition of a relatively modest amount of extra Wee1 protein (3-fold) has a significant effect on cell cycle progression.

To examine whether the Wee1-induced delay of mitosis in *Xenopus* extracts could be attributed to the increased tyrosine phosphorylation of Cdc2, we examined the phosphotyrosine content of Cdc2 at successive intervals during the cell cycle in the presence and absence of exogenous Wee1 protein (Figure 5C). In the control incubation, the tyrosine phosphorylation of Cdc2 peaked at 75 min post-activation and then abruptly declined to low levels at 90 min (Figure 5C, lanes 1–5), the time by which the extract had entered mitosis. In contrast, the phosphotyrosine content of Cdc2 in the extract containing added Wee1 protein was discernibly elevated and remained high for approximately 30 min longer (Figure 5C, lanes 6–10). In control experiments, we established that the Wee1-induced delay of the cell cycle could be abolished by the addition of okadaic acid (3 μM), demonstrating that the mitotic delay was not due to a non-specific effect upon the extract. Collectively, these results indicate that *Xenopus* Wee1, like the fission yeast Wee1 kinase, can modulate the length of interphase in a concentration-dependent manner.

Xenopus Wee1 Activity Is Highly Regulated During the Cell Cycle

Having established the basic properties of *Xenopus* Wee1, we next asked whether this enzyme is regulated during the cell cycle. To pursue this objective, we first prepared either interphase or M phase (CSF-arrested) extracts from *Xenopus* eggs. To arrest the extracts in interphase, we utilized either cycloheximide (which blocks cyclin synthesis) or aphidicolin (which imposes the replication checkpoint provided that there is a sufficiently high concentration of exogenously added DNA in the extract) (Dasso and Newport, 1990). Alternatively, interphase extracts without these inhibitory agents could be collected 50 min after activation with Ca^{2+}, well before the rise of MPF activity. After immunoprecipitation and immunoblotting of the various extracts with anti-Wee1 peptide antibodies, we observed that the electrophoretic mobility of Wee1 varied greatly during the cell cycle (Figure 6A). Specifically, the Wee1 protein from interphase extracts migrated as a 68-kDa species during SDS-gel electrophoresis irrespective of whether the extract had been

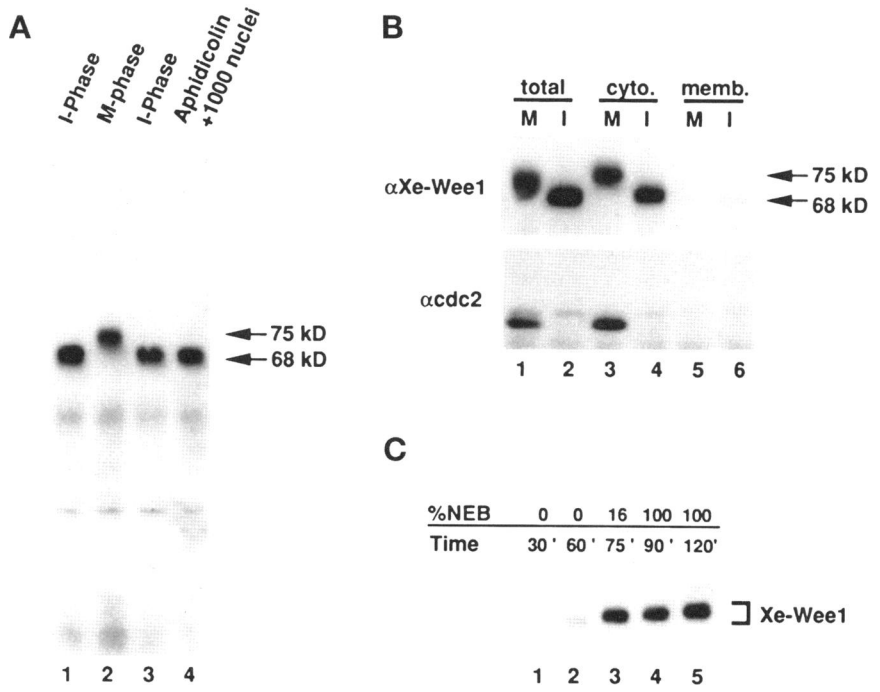

Figure 6. *Xenopus* Wee1 is localized in the cytosol and differentially modified during the cell cycle. (A) Endogenous *Xenopus* Wee1 was immunoprecipitated with anti-*Xenopus* Wee1 peptide antibodies from a CSF-arrested *Xenopus* egg extract (M-phase, lane 2), an interphase extract (I-Phase) that had been activated in the presence (lane 1) or absence (lane 3) of cycloheximide, or an S phase-blocked extract (Aphidicolin + 1000 nuclei, lane 4). Following immunoprecipitation, the samples were immunoblotted with anti-*Xenopus* Wee1 antibodies. The apparent molecular weights of the interphase and mitotic forms of *Xenopus* Wee1 (68 and 75 kDa, respectively) are indicated. (B) Total cytoplasm (lanes 1 and 2), the cytosol fraction (lanes 3 and 4), or the membrane fraction (lanes 5 and 6) were prepared from either CSF-arrested (M; lanes 1, 3, and 5) or interphase (I; lanes 2, 4, and 6) extracts as described in MATERIALS AND METHODS. The fractions were subjected to immunoprecipitation with anti-Wee1 peptide antibodies and then processed for immunoblotting with either anti-*Xenopus* Wee1 (top panel) or anti-Cdc2 (bottom panel) antibodies. Note that *Xenopus* Wee1 is found in the cytosol fraction during both M phase and interphase, but is associated with Cdc2 only during M phase. (C) A CSF-arrested *Xenopus* egg extract was activated by addition of $CaCl_2$ and aliquots were removed at the indicated times. These aliquots were bound to p13-agarose beads, washed, and processed for immunoblotting using anti-*Xenopus* Wee1 antibodies.

treated with cycloheximide, no drug, or aphidicolin (Figure 6A, lanes 1, 3, and 4). However, the M phase form of Wee1 in CSF extracts was noticeably greater in molecular mass with an electrophoretic mobility corresponding to 75 kDa (Figure 6A, lane 2). As described in greater detail below, this mobility shift is attributable entirely to phosphorylation. Upon fractionation of the egg extracts into a soluble and membrane fraction by ultracentrifugation, we observed that both the 68- and 75-kDa forms of *Xenopus* Wee1 were present almost exclusively (>90% of total) in the soluble fraction (Figure 6B).

In the course of examining the phosphorylation state of Wee1 during the cell cycle, we observed that the Cdc2 protein could be found in a complex with Wee1 in a cell cycle dependent manner. Specifically, immunoblotting of anti-Wee1 immunoprecipitates with anti-Cdc2 antibodies revealed the specific association of Cdc2 with Wee1 in M phase extracts but not interphase-arrested extracts (Figure 6B, bottom panel, compare lanes 1 and 3 with lanes 2 and 4). In time-course experiments with cycling extracts (Figure 6C), we observed that the endogenous Wee1 protein also could be bound to p13-agarose beads (an affinity absorbent for Cdc2 and associated proteins) depending upon the state of phosphorylation of the Wee1 protein. Specifically, there was no binding to p13-agarose beads by the 68-kDa, underphosphorylated form of Wee1 from extracts early in the cell cycle (<60 min post-activation). However, between 75 and 120 min post-activation, there was a preferential association of hyperphosphorylated Wee1 with p13-agarose which coincided with the timing of nuclear envelope breakdown in the extract (Figure 6C, lanes 3–5). Greater than 75% of the Wee1 protein from M phase extracts bound specifically to p13-agarose beads in these experiments (our unpublished observation).

Next, we examined whether the activity of *Xenopus* Wee1 would vary in a cell-cycle dependent manner. To measure the kinase activity of Wee1, we utilized any one of three procedures (Figure 7). For example, we could detect Wee1-dependent tyrosine phosphorylation of Cdc2 by measuring either its reactivity with anti-phosphotyrosine antibodies (Figure 7A) or its incorporation of ^{32}P from radioactive ATP (Figure 7C). Alternatively, the kinase activity of Wee1 could be assessed on the basis of its ability to phosphorylate itself in the presence of radioactive ATP (Figure 7B).

By all three assay methods, we observed a large difference in kinase activity between the 68- and 75-kDa forms of *Xenopus* Wee1. The 68-kDa interphase form of Wee1 was highly active (Figure 7A, lanes 5–8; Figure 7B, lane 2; and Figure 7C, lane 3), as might be expected for an enzyme that serves to shut off Cdc2 during this phase of the cell cycle. In contrast, the 75-kDa mitosis-specific form of Wee1 showed little if any ability to phosphorylate either Cdc2 or itself in the various kinase assays (Figure 7A, lanes 3 and 4; Figure 7B, lane 1; and Figure 7C, lane 2). Quantitation of the various assays performed indicated that the 68-kDa

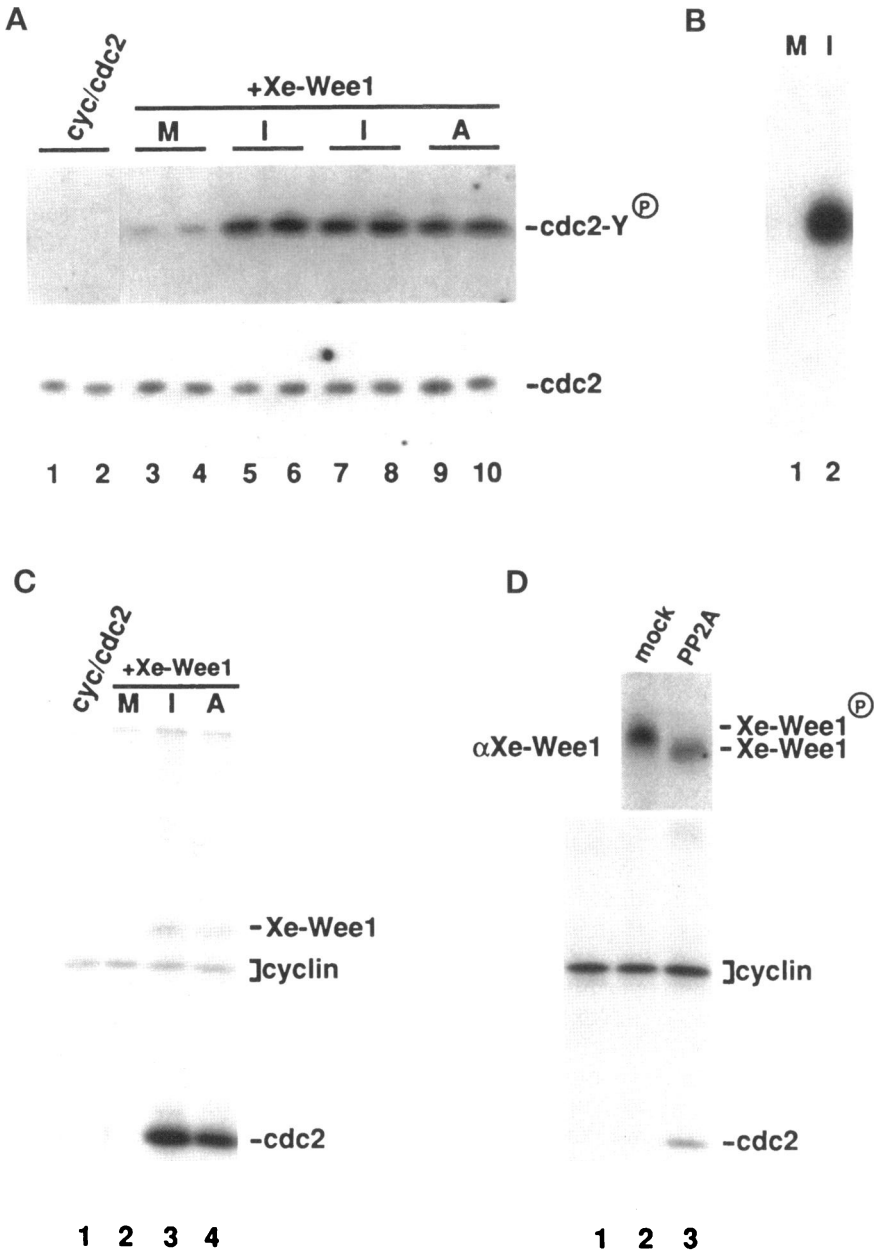

Figure 7. *Xenopus* Wee1 activity is regulated during the cell cycle by its phosphorylation state. (A) Endogenous *Xenopus* Wee1 was immunoprecipitated from a CSF-arrested *Xenopus* egg extract (M, lanes 3 and 4), an interphase extract that had been activated in the presence (I, lanes 5 and 6) or absence (I, lanes 7 and 8) of cycloheximide, or an S-phase blocked extract (A, lanes 9 and 10) that had been treated with aphidicolin in the presence of 1000 sperm nuclei per μl. The kinase activity of each of these immunoprecipitates was then measured in vitro using a purified recombinant cyclin/Cdc2 substrate in the presence of an ATP regenerating system as described in Figure 3. The reaction products were immunoblotted and probed with either anti-phosphotyrosine (top panel) or anti-Cdc2 (bottom panel) antibodies. The same *Xenopus* Wee1 immunoprecipitates used in this experiment were also immunoblotted for *Xenopus* Wee1 in Figure 6. Note that equivalent amounts of *Xenopus* Wee1 are present in all of the samples as judged by immunoblotting with anti-*Xenopus* Wee1 antibodies (see Figure 6A). (B) Auto-kinase activity of *Xenopus* Wee1. *Xenopus* Wee1 was immunoprecipitated from a CSF-arrested (M, lane 1) or an interphase (I, lane 2) *Xenopus* egg extract and incubated with [^{32}P]ATP and processed for autoradiography. (C) Recombinant human cyclin B1/*Xenopus* T161A kinase-negative Cdc2 substrate (cyc/Cdc2) was incubated with [^{32}P]ATP in the absence (lane 1) or presence (+Xe-Wee1, lanes 2–4) of endogenous *Xenopus* Wee1 which had previously been immunoisolated from either a CSF-arrested (M, lane 2), interphase (I, lane 3), or S-phase arrested (A, Aphidicolin plus 2000 nuclei per μl, lane 4) *Xenopus* egg extract. The migration of phosphorylated *Xenopus* Wee1 (Xe-Wee1), human cyclin B1 (cyclin) and *Xenopus* Cdc2 (Cdc2) have been indicated. (D) In vitro dephosphorylation of the modified *Xenopus* Wee1 protein restores its kinase activity. Immunoisolated mitotic *Xenopus* Wee1 protein (lanes 2 and 3) was treated with phosphatase buffer alone (mock, lane 2) or with the same buffer containing purified erythrocyte phosphatase 2A (PP2A, lane 3). After treatment with okadaic acid and subsequent washing, the samples were either immunoblotted using anti-*Xenopus* Wee1 antibodies (top panel) or assayed for Wee1 activity in the presence of cyclin/Cdc2 substrate and [^{32}P]ATP (bottom panel). The positions of phosphorylated and unphosphorylated *Xenopus* Wee1 (top panel) as well as cyclin and Cdc2 (bottom panel) have been indicated. Lane 1 depicts the Cdc2/cyclin complex incubated without added Wee1 protein.

form of Wee1 was 7–20-fold more active than the 75-kDa form. Finally, we demonstrated that the reduced kinase activity of the 75-kDa mitotic version of Wee1 was indeed due to phosphorylation. Specifically, we found that treatment of the 75-kDa form of *Xenopus* Wee1 with protein phosphatase 2A (PP2A) could both reverse the mobility shift back to 68 kDa (Figure 7D, top panel) and restore the ability of Wee1 to phosphorylate Cdc2 (Figure 7D, bottom panel). Consistent with this observation, we observed that treatment of *Xenopus* interphase egg extracts with okadaic acid (3 μM) resulted in the rapid phosphorylation of Wee1 (our unpublished observation).

These experiments establish that Wee1 activity is high during interphase and low during mitosis, but it is also important to ask whether the activity of Wee1 might be modulated during interphase by parameters that would be expected to contribute to the various checkpoints that

operate during the cell cycle. In the *Xenopus* egg system, it has been possible to reconstitute the replication checkpoint in vitro by the addition of DNA synthesis inhibitors (e.g., aphidicolin) to extracts containing greater than several hundred nuclear equivalents of DNA per μl of egg lysate (Dasso and Newport, 1990).

To examine whether the activity of the *Xenopus* Wee1 homolog described in this study might be modulated directly by the replication checkpoint, we immunoprecipitated Wee1 from interphase extracts prepared in the presence or absence of aphidicolin and 1000 demembranated *Xenopus* sperm nuclei per μl of egg extract. We consistently observed that neither the electrophoretic mobility (Figure 6A, lane 4) nor the kinase activity (Figure 7A, lanes 9 and 10; Figure 7C, lane 4) of this *Xenopus* Wee-like kinase was significantly altered by the presence of aphidicolin. Taken together, these results indicate that the activity of the particular Wee1 homolog examined in these studies is not affected by the imposition of the replication checkpoint, even though the extracts from which this Wee1 protein had been immunoprecipitated remain efficiently blocked in interphase due to the presence of unreplicated DNA.

Cdc2 and an Additional Kinase Phosphorylate Wee1 at Mitosis

The above experiments indicate that the *Xenopus* Wee1 protein becomes down-regulated at mitosis due to the action of one or more protein kinases that shift its electrophoretic mobility from 68 to 75 kDa. To address the identity of the Wee1-inhibitory kinase(s), we carried out a number of experiments. One line of experimentation involved the use of a monoclonal antibody (MPM-2) that recognizes a myriad of mitotic phosphoproteins (known as MPM-2 antigens) in a wide variety of cell types. The enzyme(s) responsible for the generation of the MPM-2 epitope at mitosis is known as the ME kinase (Kuang *et al.*, 1994). To address the possibility that the Wee1 protein might be an MPM-2 antigen, we subjected Wee1 protein that had been immunoprecipitated from *Xenopus* extracts to immunoblotting with MPM-2 antibodies. As shown in Figure 8A, the mitotic form of Wee1 (75 kDa) reacted strongly with MPM-2 antibodies (lane 2), but the 68 kDa form of Wee1 in interphase-arrested extracts displayed no MPM-2 reactivity (lanes 1, 3, and 4). In parallel, we examined whether the recombinant fission yeast Wee1 protein that had been introduced into *Xenopus* egg extracts could also become an MPM-2 antigen (Figure 8B). Consistent with the observations on the endogenous *Xenopus* protein, the exogenous fission yeast Wee1 protein that had been reisolated from mitotic egg extracts reacted well with the MPM-2 antibodies (Figure 8B, lane 1), whereas the form incubated in interphase extracts did not (Figure 8B, lane 2).

A number of studies have suggested that the ME kinase is distinct from the Cdc2 kinase (Kuang *et al.*, 1991, 1994; Kuang and Ashorn, 1993). However, the consensus sequences for phosphorylation by the ME or Cdc2 kinases both share the property of containing either Ser-Pro or Thr-Pro as part of the recognition motif (Westendorf *et al.*, 1994). The amino acid sequence of *Xenopus* Wee1 contains at least two stretches that correspond well to the consensus sequence for recognition by the ME kinase (see DISCUSSION), but one of these sites also resembles a potential Cdc2 phosphorylation site. In addition, there are a total of 11 Ser-Pro or Thr-Pro motifs throughout the *Xenopus* Wee1 sequence (Figure 1A).

To examine whether Wee1 could serve as a substrate for Cdc2, we incubated the active Cdc2/cyclin B complex in the presence of recombinant Wee1 protein (Figure 8). As shown in Figure 8C (bottom panel), the Wee1 protein became readily phosphorylated in the presence of active Cdc2 protein kinase, as evidenced by an upward mobility shift during gel electrophoresis (lanes 2, 4, and 6). Interestingly, the shifted form of Wee1 that had been phosphorylated by Cdc2 also became reactive with MPM-2 antibodies (see lanes 2, 4, and 6 in the top panel of Figure 8C).

To ask whether the shifted form of Wee1 that had been phosphorylated by Cdc2 in vitro had altered kinase activity, we immunoprecipitated Wee1 after it had been incubated with either active Cdc2/cyclin B or kinase-negative Cdc2T161A/cyclin B, and then assayed its ability to phosphorylate Cdc2 in a second incubation (Figure 8D). We found that Wee1 that had been phosphorylated previously by Cdc2 in vitro showed a greatly reduced ability to phosphorylate Cdc2 in a subsequent kinase assay (Figure 8D, lane 1), whereas Wee1 that had been previously incubated with inactive Cdc2 (lane 2) or buffer alone (lane 3) was fully active.

The above experiments establish that Wee1 is a substrate for Cdc2 kinase in vitro. To shed light on the contribution of Cdc2 to Wee1 regulation in vivo, we asked whether *Xenopus* extracts that lack active Cdc2 protein would retain any Wee1-specific kinase activity. For this purpose, we applied M phase extracts from *Xenopus* eggs to p13-agarose, a chromatography resin that efficiently removes Cdc2 and associated proteins (Dunphy *et al.*, 1988). Repeated chromatography of the M phase extracts on p13-agarose resulted in the essentially quantitative depletion of the Cdc2 protein (99% removal; see Figure 9C). We then added ^{35}S-labeled *Xenopus* Wee1 protein to either Cdc2-depleted extracts or control extracts that had been subjected to the same protocol with agarose beads lacking p13. We observed that the ^{35}S-labeled Wee1 protein in the control extracts was phosphorylated efficiently, consistent with the results described above (Figure 9A, top and middle panels; Figure 9B, see lanes 1 and 2). Interest-

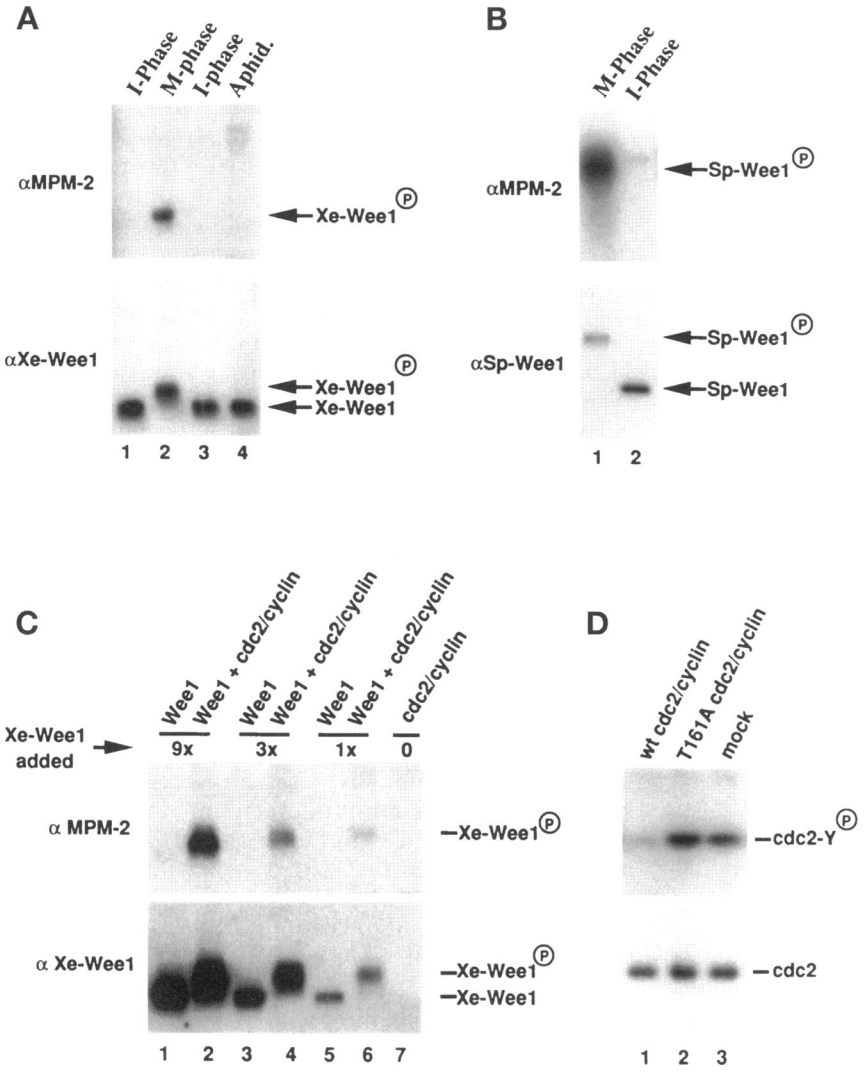

Figure 8. The Wee1 protein becomes an MPM-2 antigen at M phase, and is inactivated by in vitro treatment with active Cdc2/cyclin B. (A) Endogenous *Xenopus* Wee1 was immunoprecipitated from a CSF-arrested *Xenopus* egg extract (M-Phase, lane 2), an interphase extract (I-Phase) that had been activated in the presence (lane 1) or absence (lane 3) of cycloheximide, or an S phase-blocked extract (Aphidicolin + 1000 nuclei, lane 4). These samples were then immunoblotted and probed with either MPM-2 antibodies (top) or anti-*Xenopus* Wee1 antibodies (bottom). (B) Purified, histidine-tagged *S. pombe* Wee1 protein was added to either a CSF-arrested (M-phase, lane 1) or an interphase (I-phase, lane 2) *Xenopus* egg extract, incubated for 10 min, and then re-isolated on nickel-IDA beads. These samples were then washed, immunoblotted, and probed with either MPM-2 antibodies (top panel) or anti-*S. pombe* Wee1 antibodies (bottom panel). (C) Active Cdc2/cyclin B can phosphorylate recombinant *Xenopus* Wee1 and convert it to an MPM-2 antigen. Following an in vitro kinase reaction performed in the presence (lanes 2, 4, 6, and 7) or absence (lanes 1, 3, and 5) of wild type Cdc2/cyclin B complex, recombinant *Xenopus* Wee1 (1× = 10 ng protein per assay), was processed for immunoblotting using either anti-MPM-2 (top panel) or anti-*Xenopus* Wee1 (bottom panel) antibodies. (D) *Xenopus* Wee1 is inactivated due to phosphorylation by Cdc2/cyclin B. *Xenopus* Wee1 protein was treated with wild-type Cdc2/cyclin B (lane 1), Cdc2T161A/cyclin B (lane 2), or buffer alone (lane 3). Following the incubation, the Wee1 protein was immunoprecipitated and then assayed for the ability to phosphorylate Cdc2 as described in Figure 7A. Following the kinase assay, phosphorylation of Cdc2 was assessed with anti-phosphotyrosine antibodies (top panel) or anti-Cdc2 antibodies (bottom panel).

ingly, the Wee1 protein became phosphorylated at a similar rate in the Cdc2-depleted extract, but its electrophoretic mobility ultimately reached a plateau that was intermediate between that of the interphase and mitotic forms of Wee1 (Figure 9A, bottom panel; Figure 9B, lane 3), even after a 4 h incubation (unpublished observation). This observation indicates that Cdc2 is only partially responsible for the phosphorylation of Wee1 at mitosis, and that a distinct kinase also phosphorylates Wee1 at mitosis.

DISCUSSION

The main objective of this study was to examine the biochemical mechanisms underlying the regulation of Cdc2-specific tyrosine kinase activity in *Xenopus* egg extracts. In this system, the tyrosine phosphorylation of Cdc2 appears to be a major rate-limiting parameter controlling the entry into mitosis (Solomon *et al.*, 1990; Kumagai and Dunphy, 1991; Smythe and Newport, 1992; Lee *et al.*, 1994). Thus, the elucidation of how the Cdc2-specific tyrosine kinase (Wee1) and Cdc2-specific tyrosine phosphatase (Cdc25) activities are controlled at the molecular level will be necessary for a full understanding of mitotic initiation.

To pursue this goal, we isolated the gene for a *Xenopus* Wee1-like kinase. We established that this kinase possesses many characteristics of a Wee1 family member. First, the *Xenopus* Wee1 protein efficiently phosphorylates Cdc2 on tyrosine 15 in a manner that is completely dependent upon its association with cyclin. Second, this tyrosine phosphorylation of Cdc2 results in a substantial reduction of its kinase activity toward histone H1. Third, the elevation of Wee1 kinase levels in *Xenopus* egg extracts by the addition of recombinant Wee1 protein results in a readily observ-

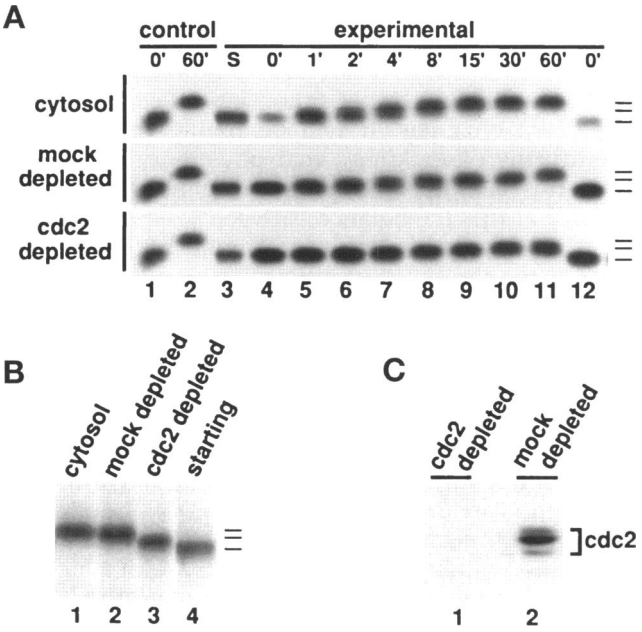

Figure 9. The *Xenopus* Wee1 protein is only partially modified in mitotic extracts which lack active Cdc2/cyclin complex. (A) ^{35}S-labeled full-length *Xenopus* Wee1 was mixed with CSF cytosol (top panel, lanes 4–12), control agarose-depleted CSF cytosol (mock depleted, middle panel, lanes 4–12), or p13-agarose depleted CSF cytosol (Cdc2 depleted, bottom panel, lanes 4–12). For reference, control lanes 1 and 2 show the migration of *Xenopus* Wee1 in the total CSF extract at 0 and 60 min, respectively. Samples were removed for electrophoresis and fluorography at the indicated times. In lane 3, the starting (S) ^{35}S-labeled *Xenopus* Wee1 has been loaded for reference. Hatch marks denote unphosphorylated (bottom mark), fully phosphorylated (top mark), and partially phosphorylated (middle mark) forms of the Wee1 protein. (B) ^{35}S-labeled full-length *Xenopus* Wee1 protein was mixed with CSF cytosol (cytosol, lane 1), control agarose-depleted CSF cytosol (mock-depleted, lane 2), or p13-agarose depleted CSF cytosol (Cdc2-depleted, lane 3). Samples were removed for electrophoresis and fluorography at 60 min. In lane 4, the starting ^{35}S-labeled *Xenopus* Wee1 has been loaded for reference. (C) The Cdc2 protein from CSF cytosol is quantitatively depleted (>99% removal) by p13-agarose chromatography. The CSF cytosol from part (A) was immunoblotted with anti-Cdc2 antibodies following treatment with either p13-agarose beads (lane 1) or control-agarose beads (lane 2).

able delay of the entry into mitosis. Finally, the *Xenopus* Wee1 protein can be found in a complex with Cdc2 at mitosis, suggesting a biochemical interaction between the two proteins.

Next, we examined the regulation of this enzyme and found that the activity of *Xenopus* Wee1 varies in a manner consistent with its role as a negative regulator of mitosis: its activity is high during interphase when it should suppress Cdc2, and low during mitosis when Cdc2 must be active. The Wee1-like kinase that we have identified is present in constant amounts throughout the first embryonic cell cycle in *Xenopus*, but undergoes a large variation in kinase activity during this period. This oscillation is accompanied by substantial changes in the phosphorylation state of *Xenopus* Wee1. Specifically, the interphase form of Wee1 (68 kDa) is highly active and does not appear to be extensively phosphorylated, as judged by its mobility during SDS-gel electrophoresis. In contrast, the mitotic form of Wee1 (75 kDa) is heavily phosphorylated and essentially inactive. The inability of the mitotic form of Wee1 to phosphorylate Cdc2 is a direct consequence of phosphorylation, since the protein can be reactivated by in vitro treatment with PP2A.

These experiments directly substantiate the existence of two types of Wee1-regulatory factors: a Wee1 inhibitory kinase(s) and a Wee1-stimulatory phosphatase(s). A variety of arguments suggest that the stimulatory phosphatase that keeps *Xenopus* Wee1 active during interphase corresponds to a form of protein phosphatase 2A. In this study, we have found that this Wee1-stimulatory phosphatase activity is highly sensitive to okadaic acid. Moreover, purified protein phosphatase 2A is very effective in reversing the inhibitory phosphorylation of Wee1 in vitro. The involvement of PP2A in the up-regulation of Wee1, combined with its well established role in the down-regulation of Cdc25 (Kumagai and Dunphy, 1992; Izumi *et al.*, 1992; Clarke *et al.*, 1993; Lee *et al.*, 1994), provides a logical mechanism for the coordinate regulation of the enzymes involved in the tyrosine phosphorylation and dephosphorylation of Cdc2 [reviewed in Dunphy (1994)].

Another important issue that we have addressed involves the identity of the Wee1 inhibitory kinase(s) that shut off Wee1 during mitosis. The *Xenopus* Wee1 coding sequence contains eleven Ser-Pro (SP) or Thr-Pro (TP) motifs that are found in the consensus recognition site for a number of mitotic kinases, including the Cdc2, MAP, and ME kinases (Nigg, 1991; Thomas, 1992; Westendorf, 1994). Interestingly, ten of these SP/TP motifs are found in the N-terminal one-third of *Xenopus* Wee1 outside of the area that comprises the catalytic domain of the kinase. A high concentration of SP/TP motifs in the N-terminal region is a characteristic shared with Wee1 proteins from other species, including fission yeast, budding yeast, and humans (Russell and Nurse, 1987; Booher *et al.*, 1993; N. Watanabe, M. Broome, and T. Hunter, unpublished observations). In the case of the fission yeast protein, this N-terminal region was shown to be phosphorylated selectively in mitotic *Xenopus* egg extracts (Tang *et al.*, 1993). A plausible model is that the N-terminal domain comprises a regulatory domain whose phosphorylation at mitosis inhibits the capacity of Wee1 to act as a kinase and/or recognize Cdc2. Interestingly, we have found in this study that the M phase form of Wee1 cannot phosphorylate either itself or Cdc2, suggesting that the catalytic mechanism of Wee1 has been impeded.

In addressing the identity of the Wee1-inhibitory kinases, we undertook a number of complementary approaches. In the most direct experiment, we asked whether highly active Cdc2/cyclin B would be able to phosphorylate the *Xenopus* Wee1 protein in a cell-free reaction. These studies demonstrated that Cdc2/cyclin B could phosphorylate Wee1 very efficiently, and that this phosphorylation resulted in a strong inactivation of Wee1 kinase activity. The involvement of Cdc2 in down-regulating Wee1 provides direct evidence for an autocatalytic or self-perpetuating feedback loop in which limited amounts of active Cdc2 can trigger the full activation of Cdc2/cyclin B (MPF) at mitosis (Solomon *et al.*, 1990; Kumagai and Dunphy, 1992; Izumi *et al.*, 1992; Smythe and Newport, 1992; Hoffmann *et al.*, 1993; Lee *et al.*, 1994). The biochemical events that lead to the initial triggering of this feedback circuit remain to be elucidated. In particular, it is not clear how the small amount of active Cdc2 that triggers this process is produced initially.

A resolution to this paradox might be provided by our observation that Cdc2 is not the sole kinase that phosphorylates Wee1 in M phase extracts. Based on the findings in this paper and an earlier study from our laboratory (Tang *et al.*, 1993), we strongly favor the idea that multiple kinases, including Cdc2 and another activity (kinase X) phosphorylate Wee1 at mitosis. For example, M phase extracts that have been depleted of active Cdc2/cyclin B by chromatography on p13-agarose beads nonetheless retain substantial kinase activity specific for *Xenopus* Wee1 (Figure 9) and fission yeast Wee1 (Tang *et al.*, 1993). Moreover, although *Xenopus* Wee1 possesses eleven SP/TP-containing motifs, only one or two of them (PITPQR, PYTPKS) closely resemble potential Cdc2 phosphorylation sites (Nigg, 1991). There are four sequences, namely PITPQR (see above), PGTPLH, PYTPKS (see above), and PFTPES, that fit the consensus for recognition by MAP kinases (Thomas, 1992). Finally, the PITPQR sequence, as well as an additional motif (LLTPVF), fit the criteria for phosphorylation by the MPM-2 epitope (ME) kinase (Westendorf *et al.*, 1994).

The MPM-2 epitope is recognized by a monoclonal antibody which reacts with a phosphopeptide moiety present on a large number of mitotic phosphoproteins, including cell cycle regulatory factors such as the Cdc25 protein (Kuang *et al.*, 1994). The parallels between Wee1 and Cdc25 regulation raised the possibility that Wee1 also might be an MPM-2 antigen. Indeed, we found that the mitotic form of Wee1 reacts strongly with MPM-2 antibodies. The identity of the ME kinase(s) has not been established, but published studies have suggested that it does not correspond to Cdc2 (Kuang and Ashorn, 1993; Kuang *et al.*, 1994) [also see Westendorf *et al.* (1994)]. Curiously, this supposition is not consistent with our finding that Wee1 phosphorylated by Cdc2 in vitro becomes an MPM-2 antigen. However, the recognition sites for Cdc2 and the putative ME kinase share common features (Westendorf *et al.*, 1994). Therefore, it is likely that there may be overlap in the phosphorylations carried out by these two enzymes. It will be highly important to identify the other kinase that phosphorylates Wee1, since this activity is an excellent candidate for the factor that tips the balance between Wee1 and Cdc25 so as to trigger the tyrosine dephosphorylation of Cdc2 at the threshold of mitosis.

In the fission yeast and frog systems, the Wee1 protein kinase has been implicated in various checkpoints that control the orderly progression of the cell cycle. In fission yeast, the Wee1 protein is clearly involved in controlling the size at which cells enter mitosis (Nurse, 1975; Russell and Nurse, 1987). Moreover, although the inactivation of Wee1 function alone in fission yeast does not perturb the DNA replication checkpoint (Enoch and Nurse, 1990), the simultaneous elimination of the Mik1 protein (a kinase that appears to be redundant with Wee1) (Lundgren *et al.*, 1991) renders cells incapable of arresting the cell cycle in the presence of replication inhibitors such as hydroxyurea. Similarly, it has been proposed that a Cdc2-specific tyrosine kinase activity in *Xenopus* extracts might be pivotal for the replication checkpoint (Smythe and Newport, 1992). The activity of the *Xenopus* Wee1-like kinase described in this paper appears not to be modulated directly by the presence of unreplicated DNA. In other studies, we have shown that total Cdc2-specific tyrosine kinase activity is not increased in aphidicolin-containing *Xenopus* egg extracts (A. Kumagai and W. G. Dunphy, unpublished data). Thus, it does not appear that the Wee1 kinase(s) in *Xenopus* extracts are directly modulated by the replication checkpoint.

In vertebrates such as *Xenopus*, Cdc2 is negatively regulated by phosphorylation on both Tyr-15 and Thr-14. The *Xenopus* Wee1 homolog, like the previously identified human Wee1 homolog (Igarashi *et al.*, 1991; Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993), clearly phosphorylates Cdc2 only on Tyr-15. This observation is consistent with the fact that *Xenopus* Wee1 is a soluble enzyme, whereas the kinase that phosphorylates Thr-14 has been reported to be tightly associated with the cellular membrane fraction (Kornbluth *et al.*, 1994). In this study, we have not addressed the intracellular localization of Wee1 (e.g., cytosolic vs. nuclear), but interestingly the sequence of Wee1 does contain one or more good candidates for a nuclear localization signal (NLS). One such region (KRLDGCFYAIKRSKK, residues 229–243) matches well the bipartite NLS found in nucleoplasmin and dozens of other nuclear proteins (Dingwall and Laskey, 1991). This sequence is found near kinase subdomains I and II and is well conserved in the human Wee1 homolog. However, since there are other basic

stretches in the *Xenopus* Wee1 sequence (KKLK, KRKER, RGRKR), the definitive identification of an NLS within *Xenopus* Wee1 must rely upon systematic mutagenesis studies.

In conclusion, we have provided an overview of how a Wee1-like tyrosine kinase specific for Cdc2 is regulated during the early embryonic cell cycle in the *Xenopus* system. Eventually, the elucidation of how the various enzymes that act upon Wee1 (e.g, the Wee1-stimulatory form of PP2A and the multiple Wee1-inhibitory kinases) are controlled will be essential to obtain a comprehensive view of how cells execute mitosis faithfully.

ACKNOWLEDGMENTS

We thank A. Kumagai for generously providing the baculovirus constructs for histidine-tagged human cyclin B1 and the various Cdc2 mutants. We are grateful to D. Morgan (University of California, San Francisco) for supplying the baculovirus transfer vector for histidine tagging. J. Kuang (M.D. Anderson Cancer Center) kindly provided MPM-2 antibodies. This work was supported by grants from the National Institutes of Health, Lucille P. Markey Charitable Trust, National Science Foundation, and Gustavus and Louise Pfeiffer Foundation. P.R.M. and T.R.C. are supported by fellowships from the Helen Hay Whitney Foundation and the National Institutes of Health (NRSA), respectively. W.G.D. is an investigator of the Howard Hughes Medical Institute.

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